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Dichotomy in Hedgehog Signaling between Human Healthy Vessel and Atherosclerotic Plaques

Karla C S Queiroz,^{1,2} Maarten F Bijlsma,² René A Tio,³ Clark J Zeebregts,⁴ Marina Dunaeva,⁵ Carmen V Ferreira,⁶ Gwenny M Fuhler,¹ Ernst J Kuipers,¹ Maria M Alves,¹ Farhad Rezaee,¹ C Arnold Spek,² and Maikel P Peppelenbosch¹

¹Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands; ²Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef, the Netherlands; ³Department of Cardiology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; ⁴Department of Surgery, Division of Vascular Surgery, University Medical Center Groningen, Groningen, the Netherlands; ⁵Nijmegen Centre for Molecular Life Sciences (NCMLS)/Institute for Molecules and Materials (IMM), Radboud University Nijmegen, the Netherlands; and ⁶Department of Biochemistry, Institute of Biology, State University of Campinas, Brazil, Campinas, São Paulo, Brazil

The major cause for plaque instability in atherosclerotic disease is neoangiogenic revascularization, but the factors controlling this process remain only partly understood. Hedgehog (HH) is a morphogen with important functions in revascularization, but its function in human healthy vessel biology as well as in atherosclerotic plaques has not been well investigated. Hence, we determined the status of HH pathway activity both in healthy vessels and atherosclerotic plaques. A series of 10 healthy organ donor-derived human vessels, 17 coronary atherosclerotic plaques and 24 atherosclerotic carotid plaques were investigated for HH pathway activity. We show that a healthy vessel is characterized by a high level of HH pathway activity but that atherosclerotic plaques are devoid of HH signaling despite the presence of HH ligand in these pathological structures. Thus, a dichotomy between healthy vessels and atherosclerotic plaques with respect to the activation status of the HH pathway exists, and it is tempting to suggest that downregulation of HH signaling contributes to long-term plaque stability.

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INTRODUCTION

Cardiovascular disease is the leading cause of death for both men and women in much of the Western world and is predicted to be the leading global killer by 2020 (1). Atherosclerosis is responsible for coronary heart disease, most strokes and limb ischemia. A large number of studies have shown thrombosis to result from plaque instability. Thrombotic events (that follow fissure or rupture of the fibrous cap with propagation of the thrombus up-

stream from the site of cap rupture) are the underlying basis for acute disease in the majority of cases involving atherosclerosis (2). Thus, understanding atherosclerotic plaque stability is highly relevant. Plaques that rupture show increased adventitial and plaque neovascularization, suggesting that neoangiogenesis contributes to their instability (3) and anti-neoangiogenic therapy is emerging as a promising novel avenue for treating plaque instability (4,5). This approach is hampered by lack of in-

sight into the activity of endogenous angiogenic signaling pathways.

Among the molecular signals involved in revascularization, the Hedgehog (HH) pathway is featured prominently (6). HH constitutes a family of highly hydrophobic secreted morphogenetic hormones of which in humans three isoforms exist: Sonic (SHH), Indian (IHH) and Desert (DHH) (7). Their functions include maintenance of the endothelial compartment and orchestrating revascularization upon vessel occlusion-induced ischemia (8). After delivery to target cells, HH binds to its transmembrane receptor Patched-1 (PTCH1). Under HH-unligated conditions, PTCH1 keeps a second transmembrane receptor called Smoothened (SMO) inactive (9). In mammals, this probably involves translocation of a vitamin D₃-like 3- β hydroxysteroid (10). After binding of HH to PTCH1, this inhibition is relieved and SMO subsequently initi-

Address correspondence to Maikel Peppelenbosch, Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, Gravendijkwal 230, NL-3015 CE Rotterdam, the Netherlands. Phone: +31-10-7032792; Fax: +31-10-7032793. E-mail: m.peppelenbosch@erasmusmc.nl.

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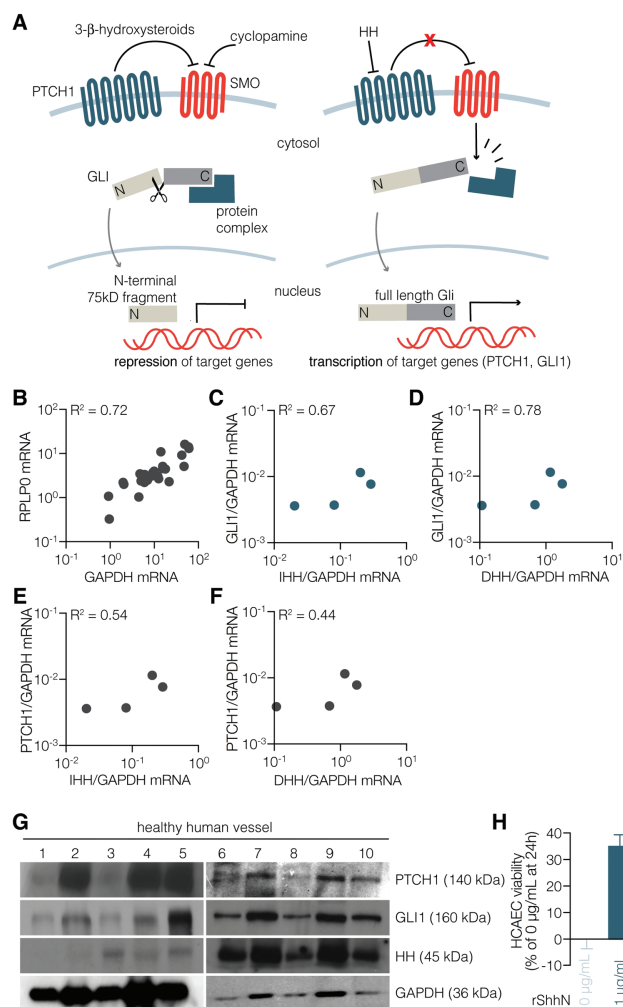


Figure 1. High activity of the HH pathway is characteristic of healthy human vessels. (A) Schematic depiction of HH signal transduction in the absence (left panel) and presence of ligand (right panel). (B) Comparison of *GAPDH* and *RPLP0* expression levels in healthy vessels and plaques. HH pathway target genes *GLI1* (C, D) and *PTCH1* (E, F) transcript levels were correlated with the expression levels of *IHH* (C, E) and *DHH* (D, F) in renal arteries from four healthy organ donors. (G) HH pathway activity in renal arteries from 10 healthy organ donors (sequentially numbered) was assessed using Western blot for the target genes. (H) Human coronary endothelial cells were cultured according to routine procedures for 24 h with either vehicle control or recombinant ShhN (1 μ g/mL). Cell viability was evaluated by MTT reduction. Data are means \pm standard error of the mean ($n = 3$) ($p < 0.05$). Because expression levels of the routinely used *GAPDH* housekeeping gene may be changed depending on HH pathway activity, we first compared *GAPDH* levels with *RPLP0* levels, since the latter gene was suggested to be a better housekeeping gene when studying HH pathway activity (27). As shown in (B), *GAPDH* and *RPLP0* levels correlate well in all samples used for this study, and we thus considered *GAPDH* an appropriate housekeeping gene.

ates signaling through the glioma-associated (GLI) transcription factors, resulting in the transcription of target genes, including both *GLI1* and *PTCH1*

(Figure 1) (10). The *in vivo* relevance of the induction of these genes was proven in ischemia experiments, in which intramyocardial gene transfer of *SHH* pro-

moted recovery and preservation of left ventricular function in myocardial ischemia by enhanced neovascularization (11), supporting the notion that HH aids in ischemic tissue rescue and especially in its revascularization. In agreement, HH production is critical for VEGF production (9,12) and endothelial maintenance and growth (9,13–17). From this, we can infer that HH signaling may well play a detrimental role as well, causing revascularization-mediated plaque instability, and constitutes an attractive candidate for targeting pathological neovascularization (18). Importantly, the activity of the HH pathway in atherosclerotic structures has not been investigated.

These considerations prompted us to investigate the activity of the HH pathway in healthy human vessels as well as in atherosclerotic pathology. We found that normal vessels are characterized by substantial HH pathway activity in response to ligand present in this tissue, but that in atherosclerotic plaques, this activity is downregulated, leading to a functional dichotomy. We propose that the downregulation of HH activity in plaques, despite the presence of ligand, may serve as a protective response of the body to mitigate the risk posed by atherosclerotic disease by enhancing plaque stability.

MATERIAL AND METHODS

Clinical Material

To obtain healthy vessels, slices of renal arteries from family kidney transplantation donors were stored in cold preservation solution, and vessels were prepared by removing connective tissue, fat and lateral vessels. After opening each vessel lengthwise, the adventitia side was dried with sterile gauze. One layer of the gauze was left on the adventitia side and cut off around the vessel, after which material was stored at -80°C . Carotid plaques were obtained from subjects undergoing carotid endarterectomy for symptomatic high-grade stenosis of the internal carotid artery. Plaques were freshly harvested and divided into sec-

tions of 5-mm thickness and stored at -80°C . Percutaneous coronary atherectomy was performed in *de novo* lesions by means of a pullback atherectomy catheter (Arrow, Reading, PA, USA). Briefly, this 10-french compatible over-the-wire catheter is positioned beyond the lesion to treat. After the cutting blade is exposed and on-axis high-speed rotation is started, the catheter is gently withdrawn by manual pullback. This procedure allows complete circumferential cuts along the whole length of the lesion, thus providing *in vivo* plaque samples, suitable for analysis. In all patients, one biopsy was taken out of the culprit lesion. All studies were done according to national and institutional ethical guidelines.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

RNA isolation from clinical samples was performed using TRIzol according to the manufacturer recommendations. The RNA was dissolved in 30 μL RNase-free water and stored at -80°C . RNA concentration was checked using a Nanodrop spectrophotometer (Isogen, Life Science, De Meern, the Netherlands). For cDNA synthesis, 1 μg RNA was used with an end volume of 20 μL . Subsequently, quantitative polymerase chain reactions (PCRs) detecting the expression level of different proteins were performed on a Roche LightCycler 480 system using the following primers ("h" stands for "human"): *hRPLP0* (*RPLP0*: ribosomal protein, large, P0) forward, GGCAC CATTG AAATC CTGAG TGATG TG; *hRPLP0* reverse, TTGCG GACAC CCTCC AGGAAGC; *hGAPDH* forward, AAGGT GAAGG TCGGA GTCAA C; *hGAPDH* reverse, TGGAA GATGG TGATG GGATT; *hGLI1* forward, GTTCA CATGC GCAGA CACAC T; *hGLI1* reverse, TTCGA GGCGT GAGTA TGACT T; *hPTCH1* forward, CGGCA GCCGC GATAA G; *hPTCH1* reverse, TTAAT GATGC CATCT GCATC C; *hSHH* forward, GCTCG GTGAA AGCAG AGAAC; *hSHH* reverse, CCAGG AAAGT GAGGA AGTCG; *hIHH* forward, CACCC CCAAT TACAA TCCAG;

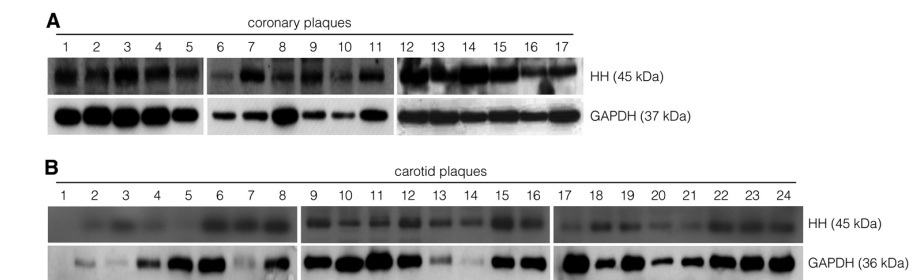


Figure 2. Abundant presence of HH ligands in atherosclerotic plaques. (A) Coronary plaques were collected from 17 patients by catheterization, and protein levels of HH ligands were analyzed by Western blot. (B) Carotid plaques were collected by endarterectomy and analyzed for the levels of HH protein using Western blot. Immature forms (as deduced from apparent molecular weight) are abundantly present in plaques. Carotid plaques also contain abundant HH. Thus, atherosclerotic plaques are characterized by the presence of HH ligands.

hIHH reverse, CCGTC TGATG TGGTG ATGTC; *hDHH* forward, ACCAA TCTAC TGCCC CTGTG; *hDHH* reverse, GTTGT AGTTG GGCAC GAGGT.

Cell Culture and MTT Assay

Primary human coronary arterial endothelial cultures (HCAECs) were maintained per provider suggestions (Lonza, Verviers, Belgium). In short, HCAECs were grown in EGM-2MV medium (Lonza, Verviers, Belgium) supplemented with 5% fetal calf serum, growth factors, hydrocortisone and gentamicine, at 37°C in a 5% CO_2 humidified atmosphere. After treatment with recombinant human Shh-N (R&D Systems, Minneapolis, MN, USA), HCAEC viability was determined by a routine 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Queiroz *et al.* (13).

Western Blotting Analysis

Plaques and healthy vessels were lysed in lysis buffer (50 mmol/L Tris [Tris(hydroxymethyl)aminomethane]-HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA [ethylene glycol tetraacetic acid], 1 mmol/L *o*-vanadate, 1 mmol/L NaF and protease inhibitors [1 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin and 1 mmol/L 4-[2-amino-ethyl]-benzolsulfonyl-fluoride-hydrochloride]) on ice. Subsequently, samples were cleared by centrifugation

and dissolved in an equal volume of 2 \times sodium dodecyl sulfide (SDS) gel loading buffer (100 mmol/L Tris-HCl [pH 6.8], 200 mmol/L dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and boiled for 5 min. Thereafter, proteins were resolved on molecular size using SDS polyacrylamide gel electrophoresis and analyzed by Western blot essentially as described previously (19). Briefly, membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)/0.1% Tween 20 (TBST) and incubated overnight at 4°C with the appropriate primary antibody. Primary antibodies against GLI1 (#2553) and PTCH1 (#2468) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-HH (5E1; recognizing IHH, DHH and SHH) (20) antibody was purchased from the Developmental Hybridoma Bank (Iowa City, IA, USA), respectively. After incubation, membranes were washed in TBST and incubated with anti-rabbit, anti-goat and anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1:2,000 dilutions in blocking buffer for 1 h. Blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland). Despite the absence of calcium or zinc salts from our buffers (21), we observed efficient immunodetection of HH ligands using the 5E1 antibody, as also shown previously by Lee *et al.* (22) and Kohtz *et al.* (23).

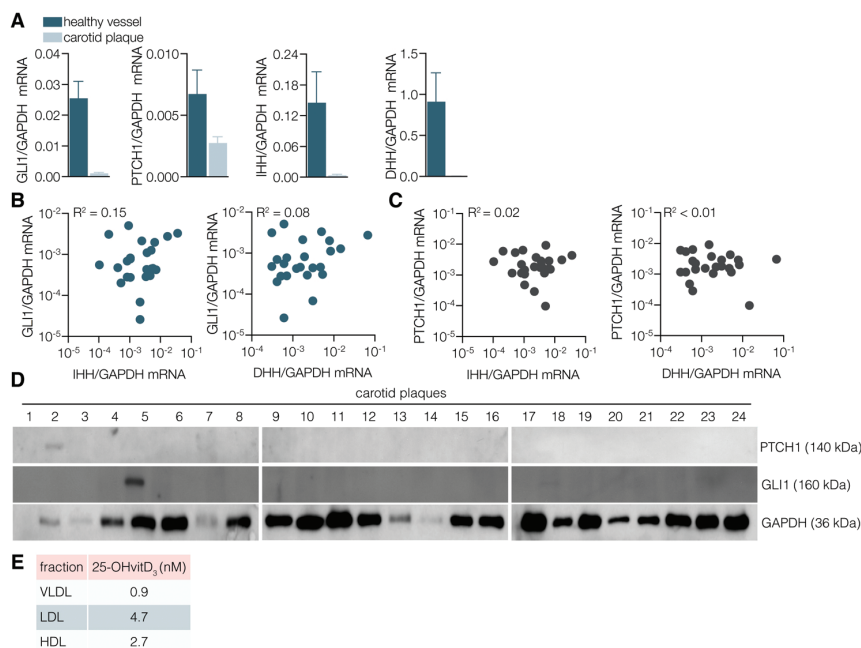


Figure 3. Absence of response to HH ligands in atherosclerotic plaques. (A) Atherosclerotic plaques were collected from 24 patients undergoing carotid endarterectomy for symptomatic high-grade stenosis of the internal carotid artery. Renal arteries from four family kidney transplantation donors were used as healthy control vessels. HH ligand expression and pathway activity was assessed by quantitative reverse transcriptase PCR. Expression of HH pathway targets *GLI1* (B) and *PTCH1* (C), as determined by quantitative reverse transcriptase PCR, was correlated with the expression level of HH ligands in atherosclerotic plaques. (D) The low levels or absence of HH target gene products were confirmed by Western blotting analysis in the same 24 subjects. (E) Radioimmunoassay analysis of three major plasma lipoproteins reveals that LDL contains the highest concentrations and VLDL the lowest levels of the endogenous HH signaling antagonist 25-OH vitamin D₃ (25-OHvitD₃).

Analysis of Vitamin D₃ in Lipoproteins

Isolation of lipoproteins was performed as described by Queiroz *et al.* (13), and radioimmunoassay for measurement of vitamin D₃ was performed as described earlier (24–26).

RESULTS

Healthy Human Vessel Is Characterized by Strong Constitutive Activation of HH Signaling

The vascular network is widely recognized to be targeted by HH ligands, since these morphogens constitute an important angiogenic signal after ischemic stress (11), but the activation status of the HH pathway in otherwise unchallenged healthy human vessel remains uncertain.

Hence, we isolated the renal artery from healthy multiorgan donors and investigated these for HH signaling activity by assessing relative mRNA expression levels of the HH pathway targets *PTCH1* and *GLI1*. Because expression levels of the routinely used *GAPDH* housekeeping gene may change depending on HH pathway activity, we first compared *GAPDH* levels with *RPLP0* levels, since the latter gene has been suggested to be a better housekeeping gene when studying HH pathway activity (27). As shown in Figure 1B, *GAPDH* and *RPLP0* levels correlate well in all samples used for this study, and we thus considered *GAPDH* to be an appropriate housekeeping gene.

Healthy vessels showed high expression of *GLI1* and *PTCH1*, and these ex-

pression levels positively correlated with the expression levels of *IHH* and *DHH* (Figures 1C–F). *SHH* transcript levels were below detection levels. To confirm these observations, we analyzed *PTCH1* and *GLI1* protein levels in 10 healthy vessels by Western blot analysis (Figure 1G). *PTCH1* and *GLI1* protein levels were easily detectable in healthy vessels, although we did observe large interpatient variability. A subsequent study in aortic, carotid and femoral artery segments (29 samples obtained during autopsy) showed that the HH ligand was abundant in all arteries studied (data not shown). Interestingly, pharmacological inhibition of HH signaling using either cyclopamine (28) or vitamin D (29) was previously shown to compromise survival of primary coronary artery endothelial cells (13), suggesting that HH signaling is not only active in this cell type, but also required for endothelial maintenance. In line with these data, treatment of primary coronary artery endothelial cells with recombinant sonic hedgehog N-terminus (ShhN) induced increased cell viability relative to control treated cells (Figure 1H). This finding confirms a recent report showing that HH signaling is important for vascular integrity in the murine coronary circulation (30) and suggests that activation of HH signaling is a general feature of the human vasculature.

Presence of HH Ligand in Atherosclerotic Plaques

Subsequently, we investigated the presence of HH ligands in atherosclerotic structures. To this end, material obtained from 17 coronary plaques was collected and subjected to Western blot analysis for HH ligand (Figure 2A). This analysis revealed local production of HH during atherosclerotic plaque formation.

The presence of HH ligand was confirmed by Western blot analysis on atherosclerotic carotid plaques (Figure 2B). Thus, locally produced HH ligand is present in substantial amounts in atherosclerotic structures in human vessels,

raising questions about the activity of HH signal transduction in atherosclerotic plaques and how plaque stability can be maintained despite the neoangiogenic influence expected from the high levels of HH ligands detected in plaques.

Absence of HH Signaling Despite Ligand Expression in Atherosclerotic Plaques

We investigated whether the presence of HH ligand in atherosclerotic plaques correlated with HH pathway activity. To this end, atherosclerotic carotid plaques obtained from 24 patients were investigated for *PTCH1* and *GLI1* expression. Interestingly, *PTCH1* and *GLI1* expression (Figure 3A) was very low in atherosclerotic plaques, especially when compared with healthy vessels. More importantly, the expression levels of *PTCH1* and *GLI1* did not correlate with the expression levels of HH ligands, as shown in Figures 3B and C. In agreement, Western blot analysis indicated that these samples contain undetectable levels or only weak traces of HH pathway targets (Figure 3D). Thus, despite the presence of abundant HH ligand in atherosclerotic plaques, this presence does not translate into activation of the HH pathway. From this result, we can infer the existence of mechanisms that limit activation of this signaling in plaques.

Lipoproteins are important constituents of atherosclerotic plaques and are potentially important carriers of endogenous HH antagonists (31). Consequently, we investigated the presence of vitamin D3 in the different lipoprotein components of the blood of healthy volunteers (Figure 3E). Interestingly, low-density lipoprotein (LDL) contains significant levels of Smoothed inhibitory vitamin D3. Because LDL is important in plaque formation, it is possible that the thus-derived local availability of vitamin D3 (or alternative hydroxysterols, which inhibit HH signaling irrespective of the presence of HH [29]) exerts a local negative effect on HH signaling, explaining the remarkable dichotomy in HH signaling between healthy vessels and athero-

sclerotic plaque. Because HH is an important mediator of revascularization and revascularization is the major cause of plaque instability, it is possible that the lipoprotein compartment exerts multilevel influence on the pathological consequences of thrombotic complications. Hence, we propose that enhanced understanding of the interaction between HH signaling and vascular biology will substantially contribute to our comprehension of the pathogenesis of cardiovascular disease.

DISCUSSION

Although most people develop atherosclerotic plaque from a young age (32), most often these plaque do not rupture. Among the factors that predispose individuals to rupture are neovascularization, and thus understanding the mechanisms that control such neoangiogenesis are important. HH signaling is among the most important signals controlling neoangiogenesis, and this consideration prompted us to investigate HH signaling in both healthy vessels and atherosclerotic plaque. We observed strong constitutive activation of HH signal transduction in healthy human vessels, which is required for survival of primary human endothelial cultures. This result is in perfect agreement with the observation that HH is important for the maintenance of the endothelial compartment *per se* in experimental rodents (30). Importantly, however, activation of HH signaling seems to be dichotomous; in atherosclerotic plaques, such signaling is not observed. Because the absence of HH signaling in plaques is expected to inhibit revascularization of such plaques, we propose that the inhibition of HH signaling in plaque material contributes to plaque stability and constitutes a protective signal (33).

CONCLUSION

The mechanism maintaining low activity of HH signaling in plaque remains unclear, but does not involve the absence of HH ligand, since ample amounts of HH protein and RNA are detected in

atherosclerotic structures. Interestingly, we did observe that (especially on the mRNA level) HH levels in healthy vessels are increased compared with atherosclerotic plaques. One could thus argue that the amount of HH in plaques is not enough to robustly activate the pathway. However, such a threshold hypothesis is not likely for morphogens that are expected to act in response to low gradients of ligand. More likely, the low activity of HH signaling in plaques must constitute inhibition downstream of the primary HH receptor Patched (7,9). Smoothed antagonists can inhibit HH signal transduction downstream of Patched and are thus obvious candidates to participate in the HH signaling dichotomy. As lipoproteins constitute important components of the plaque structure, the presence of HH antagonists in lipoproteins may negatively regulate HH pathway activity. Interestingly, we did find high levels of the Smoothed antagonist 25-OH vitamin D3 in LDL. It is tempting to propose that LDLs are Janus-faced particles in atherosclerotic disease. On one hand, LDLs contribute to plaque formation, but on the other hand, they contribute to plaque stability by carrying antagonists of downstream HH signaling. It is important, however, to point out that other mechanisms, such as suppressor of fused (SUFU) expression (7,34), may also contribute to the functional dichotomy in HH signaling in the human vasculature. Disregarding the exact mechanism of HH signal transduction inhibition in atherosclerotic plaques, it is tempting to suggest that this inhibition constitutes an important antipathogenic signal and that enhanced knowledge of the mechanisms involved may contribute to better treatment and prevention of atherosclerotic disease.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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